Supplemental Material to:

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MTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFEB

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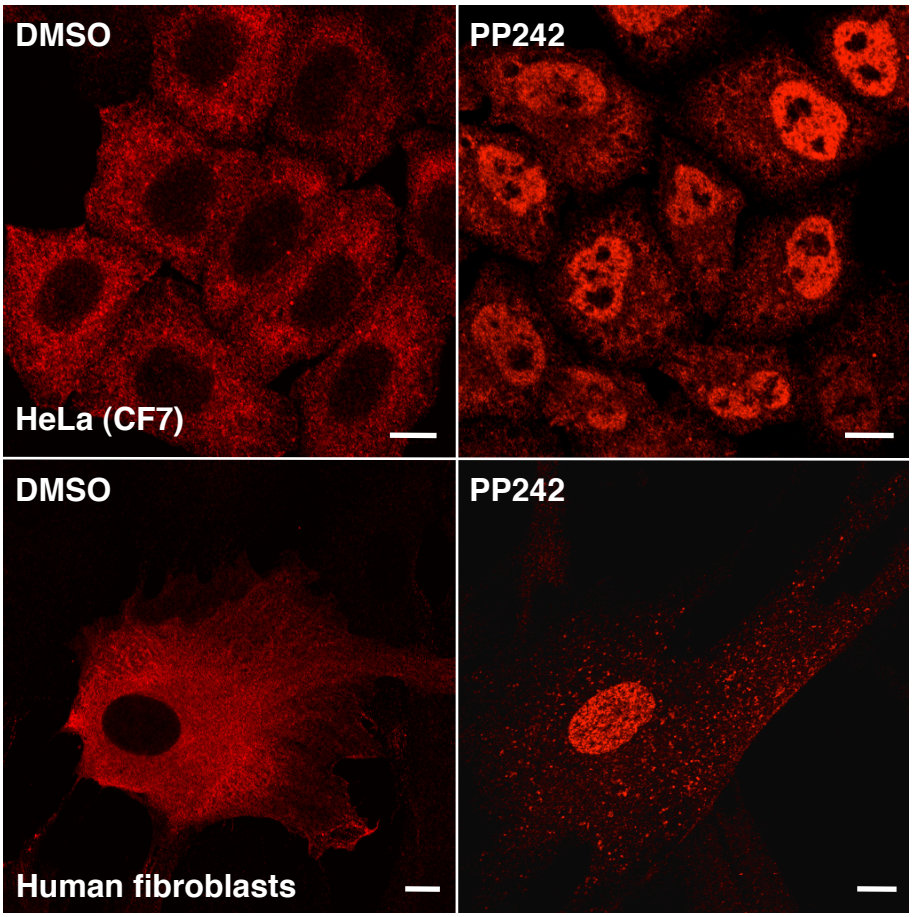


Figure S1. TFEB is redistributed to the nucleus of HeLa cells and human fibroblasts upon MTORC1 inactivation. HeLa (CF7) cells stably expressing TFEB-Flag and human fibroblast infected with adenovirus expressing TFEB-Flag were incubated with either DMSO (vehicle) or PP242 for 2 h. Cells were then washed, fixed, permeabilized with 0.5 % Triton X-100, and stained with antibodies against Flag. Scale bars, 10 μm.

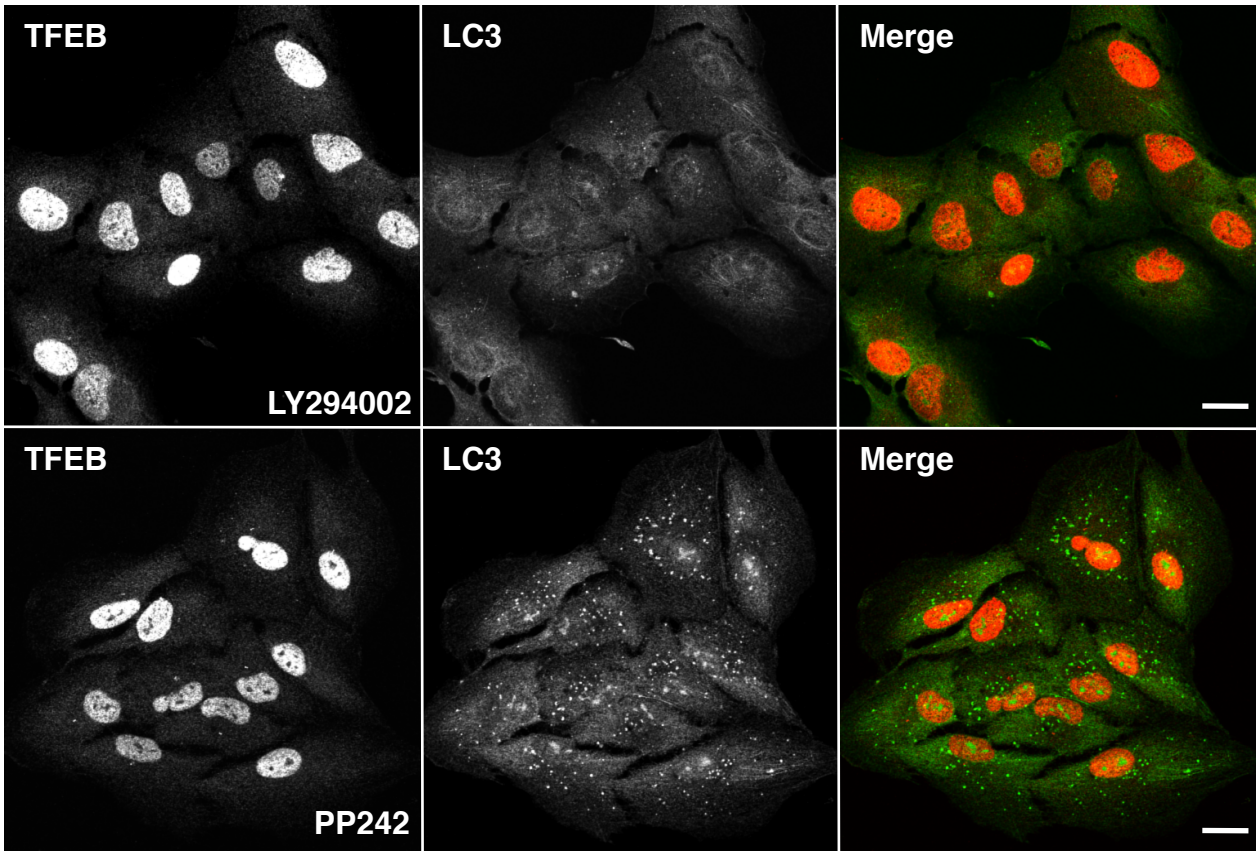


Figure S2. Delivery of TFEB to the nucleus does not require formation of autophagosomes. ARPE-19 cells were infected with adenovirus expressing TFEB-Flag. Twenty hours after infection, cells were treated with the MTORC1 inhibitors LY294002 (50 μM) or PP242 (1 μM) for 2 h, fixed in methanol:acetone, and double-stained with antibodies against Flag and LC3. Scale bars, 20 μm.

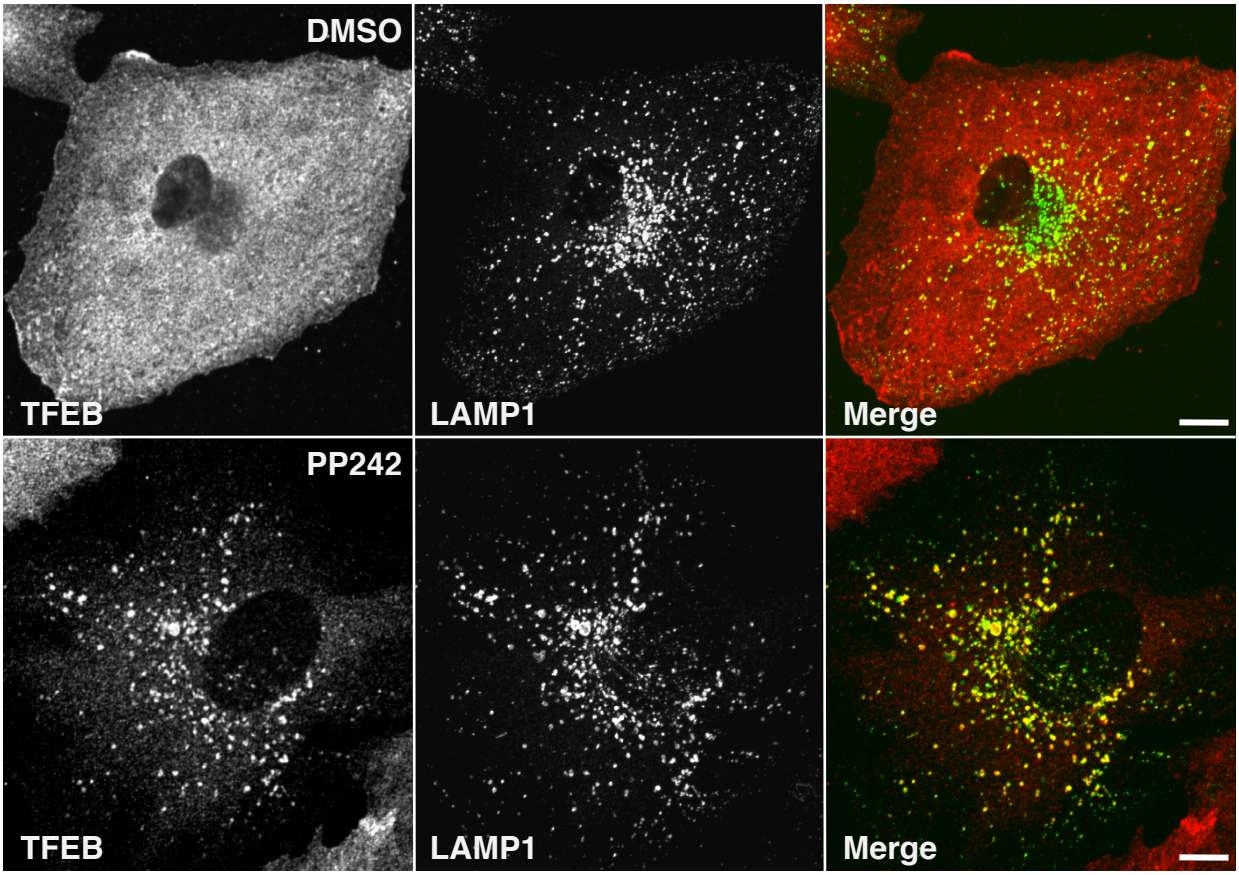


Figure S3. TFEB is recruited to late endosomes/lysosomes upon MTORC1 inactivation.
ARPE-19 cells were infected with adenovirus expressing TFEB-Flag. Twenty hours after infection, cells were incubated with either DMSO (vehicle) or PP242 for 2 h. Cells were then washed, fixed, permeabilized with 0.2 % saponin, and stained with antibodies against Flag and LAMP1. Note that permeabilization with saponin is required to preserve LAMP1 staining.
However, saponin does not permeabilize the nuclear membrane efficiently and the nuclear accumulation of TFEB upon MTORC1 inactivation can not be observed under these conditions.
Scale bars, 10 μm.

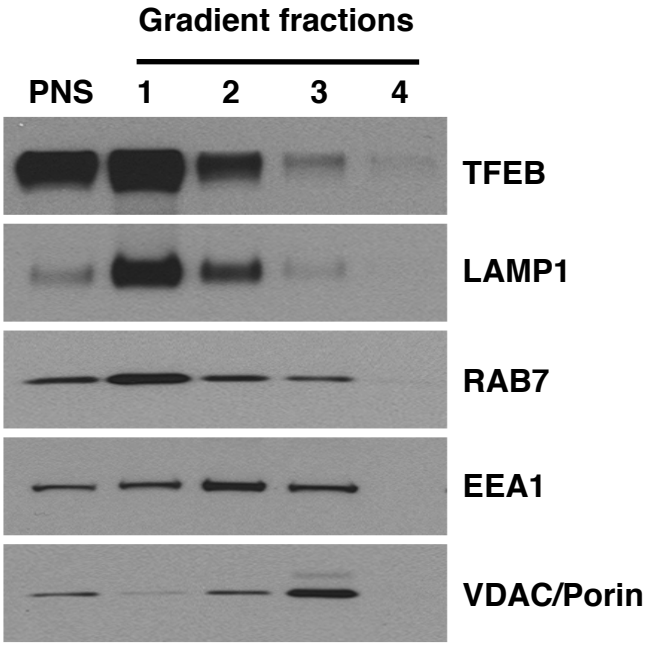


Figure S4. TFEB associates with late endosomal/lysosomal membranes. ARPE-19 cells were infected with adenovirus expressing TFEB-Flag. Twenty hours after infection, cells were homogenized and subjected to low speed centrifugation to obtain a postnuclear supernatant (PNS). Lysosomal membranes were isolated from PNS using the Lysosome Enrichment Kit for Tissue and Cultured Cells (Thermo Scientific) as described in Methods. The distribution of different membrane organelle markers and TFEB were assessed by immunoblotting using antibodies against LAMP1 (late endosomes/lysosomes), RAB7 (late endosomes), EEA1 (early endosomes) and VDAC/Porin (mitochondria).

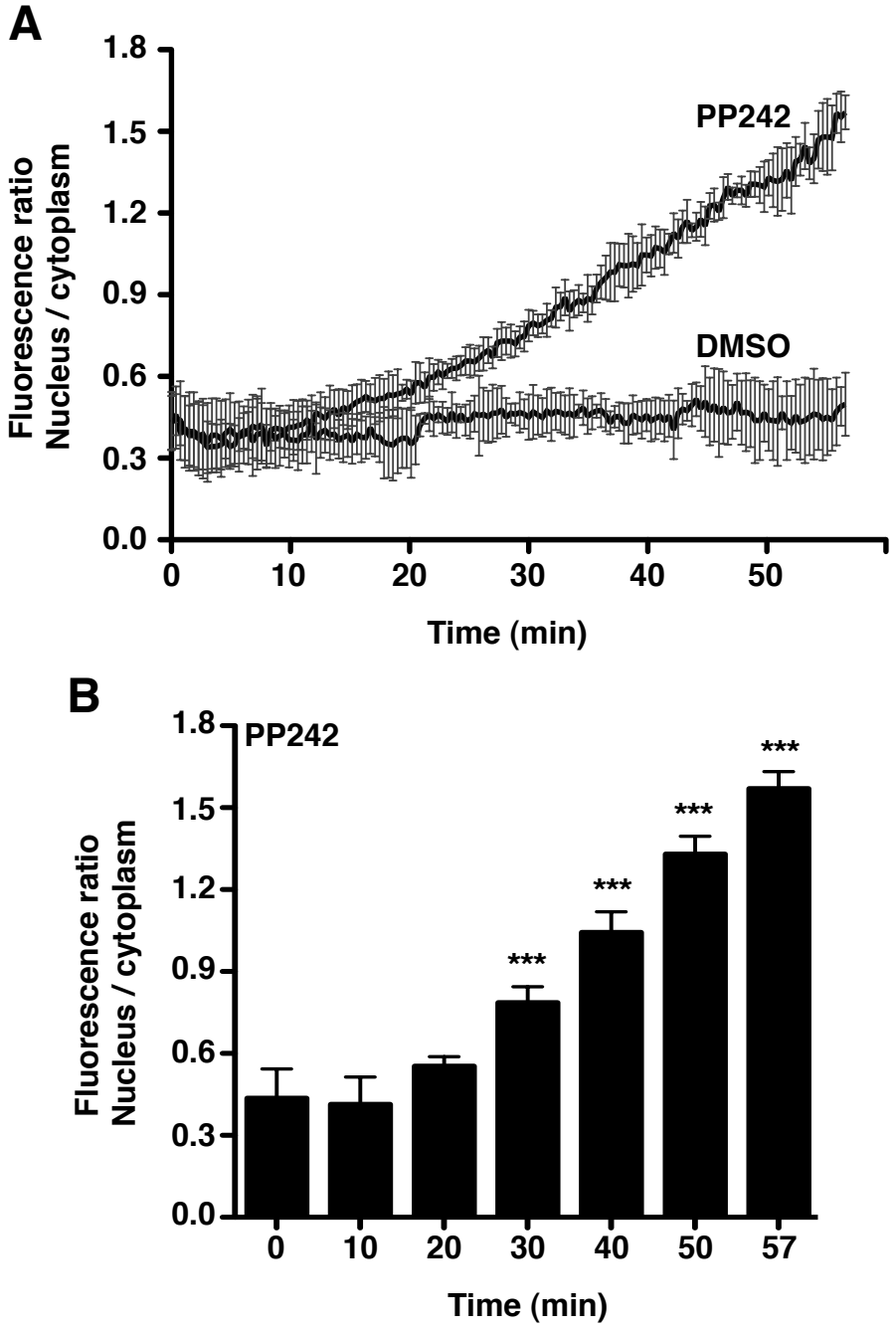


Figure S5. TFEB shuttles to the nucleus upon MTORC1 inactivation. (A) Time-lapse confocal microscopy of ARPE-19 cells transiently expressing TFEB-GFP was performed in medium containing either DMSO (vehicle) or PP242. Confocal images were acquired every 20 s for a duration of 57 min. Images were processed using NIH ImageJ software. The same size regions were used to measure the fluorescence intensities in the nucleus and cytoplasm. The graph shows quantification of the fluorescence ratio (nucleus/cytoplasm) over time. (**B**) Values at the indicated time points in (A) were plotted to show that, at approximately 30 min of incubation with PP242, TFEB-GFP was significantly accumulated into the nucleus. Values are means +/- S.D. from three independent experiments. (***)$p<0.001$.

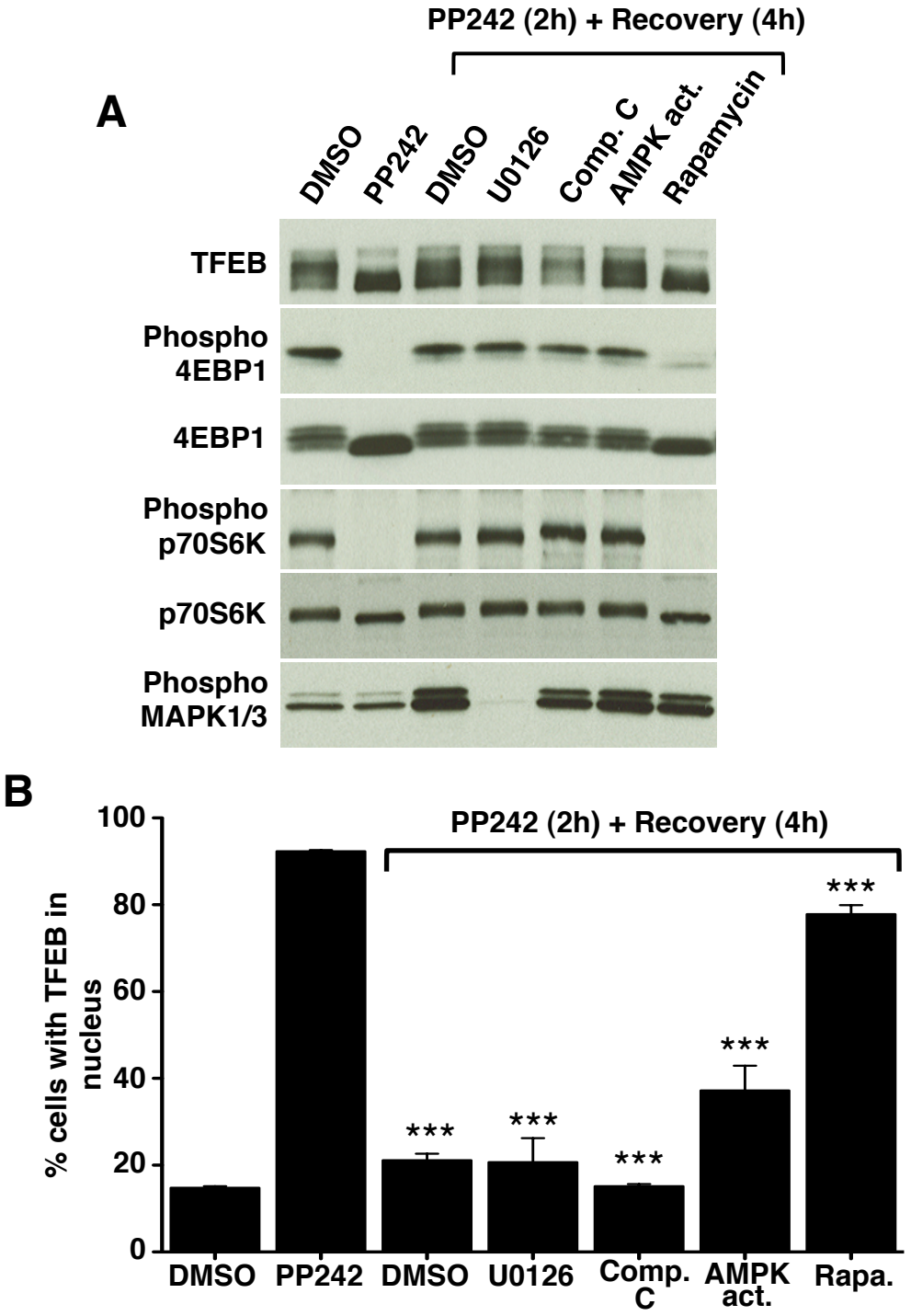


Figure S6. The changes in TFEB electrophoretic mobility induced by MTORC1 inactivation are reversible. (A) ARPE-19 cells were infected with adenovirus expressing TFEB-Flag. Twenty hours after infection, cells were incubated with either DMSO (vehicle) or PP242 for 2 h. Cells were then extensively washed, and recovered in normal medium containing the indicated drugs for 4 h. Cells were lysed and samples were subjected to immunoblotting using the indicated antibodies. **(B)** ARPE-19 cells were infected and incubated with different drugs as described in (A). Cells were then washed, fixed, permeabilized with 0.2 % Triton X-100, and stained with antibodies against Flag to assess the percentage of cells with nuclear localization of TFEB. Values are means +/- SD of three independent experiments. (***)$p<0.001$ to cells treated with PP242 for 2 h.

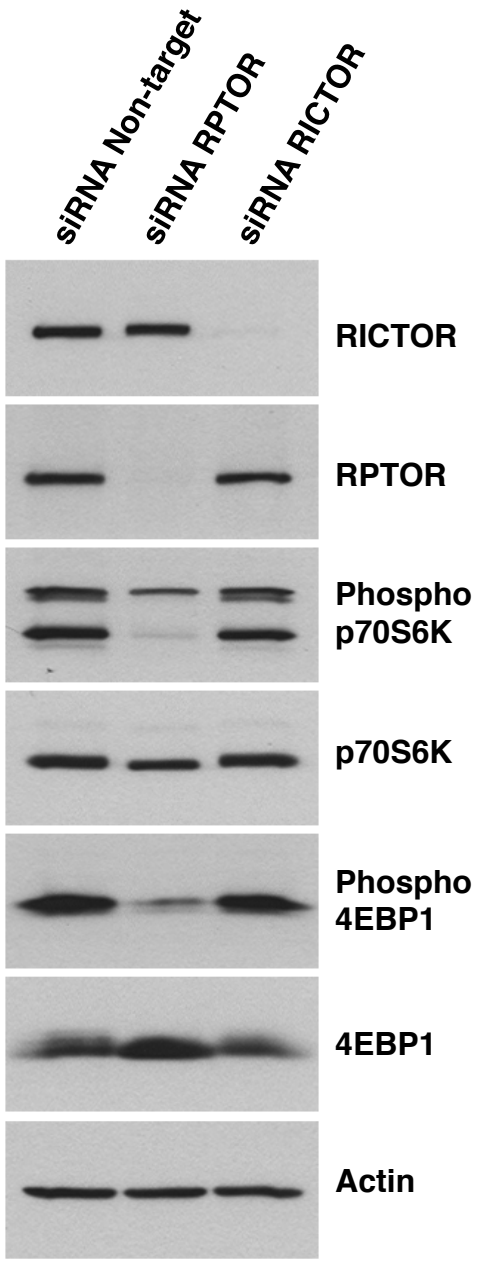


Figure S7. MTORC1 is inactivated in cells depleted of RPTOR. ARPE-19 cells were transfected with siRNA duplexes to either RPTOR or RICTOR or non-target. Seventy two hours after transfection the cells were lysed and subjected to immunoblotting using the indicated antibodies. Actin was used as a loading control.

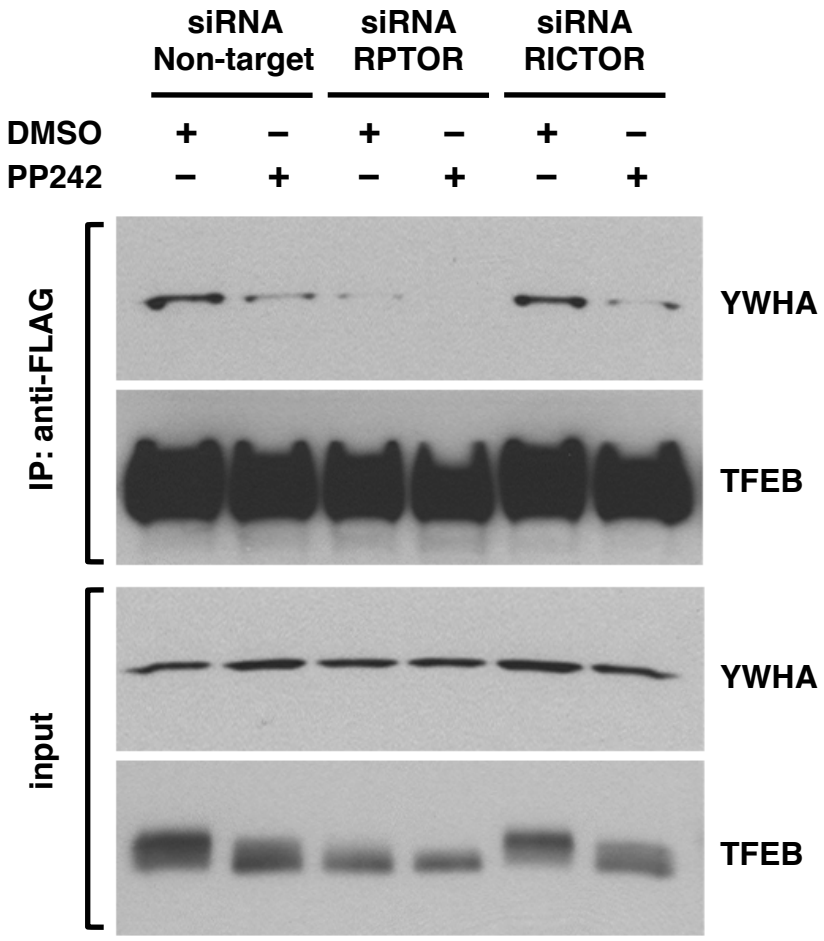


Figure S8. Inactivation of MTORC1 dissociates the TFEB/YWHA complex. ARPE-19 cells were transfected with siRNA duplexes to either RPTOR or RICTOR or non-target. Sixty hours after transfection cells were infected with adenovirus expressing TFEB-Flag. Twenty hours later the cells were incubated with either DMSO (vehicle) or PP242 for 2 h and lysed. TFEB-Flag was immunoprecipitated from cell lysates using anti-Flag antibodies. Immunoprecipitates were then subjected to immunoblotting using the indicated antibodies. Note that input represents 5% of the total cell lysate.

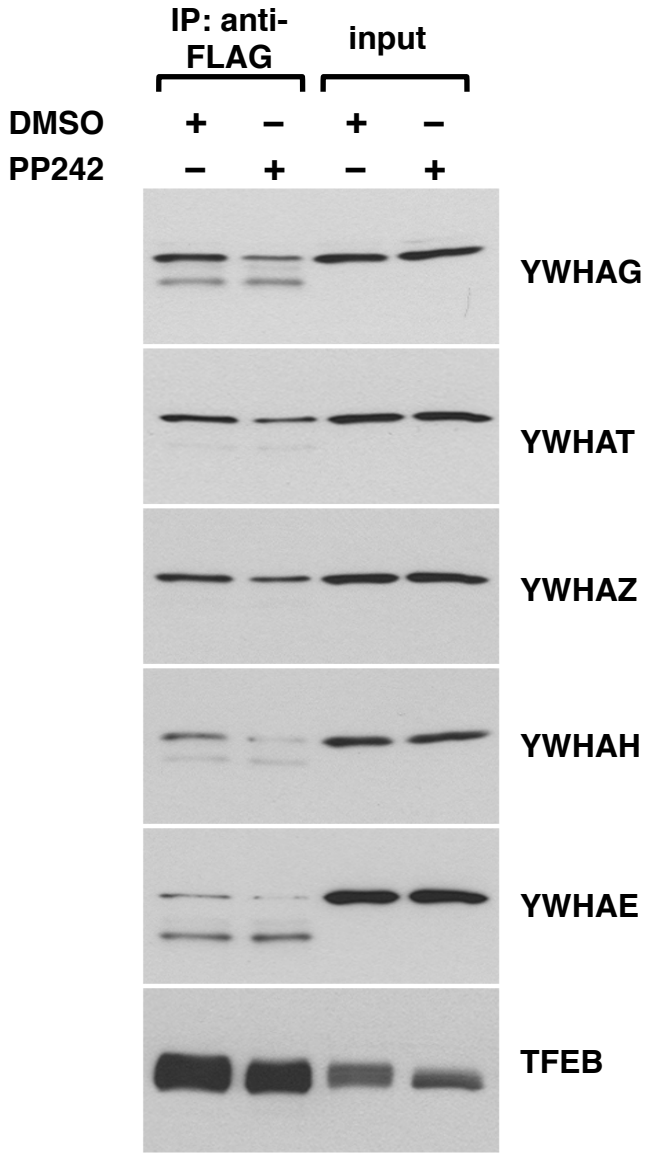


Figure S9. TFEB interacts with several YWHA isoforms. ARPE-19 cells were infected with adenovirus expressing TFEB-Flag. Twenty hours later the cells were were incubated with either DMSO (vehicle) or PP242 for 2 h and lysed. TFEB-Flag was immunoprecipitated from cell lysates using anti-Flag antibodies. Immunoprecipitates were then subjected to immunoblotting using the indicated antibodies. The followings are the YWHA (14-3-3) isoforms analyzed: YWHAG (14-3-3 gamma), YWHAT (14-3-3 Tau), YWHAZ (14-3-3 zeta), YWHAH (14-3-3 eta) and YWHAE (14-3-3 epsilon). Note that input represents 5% of the total cell lysate.

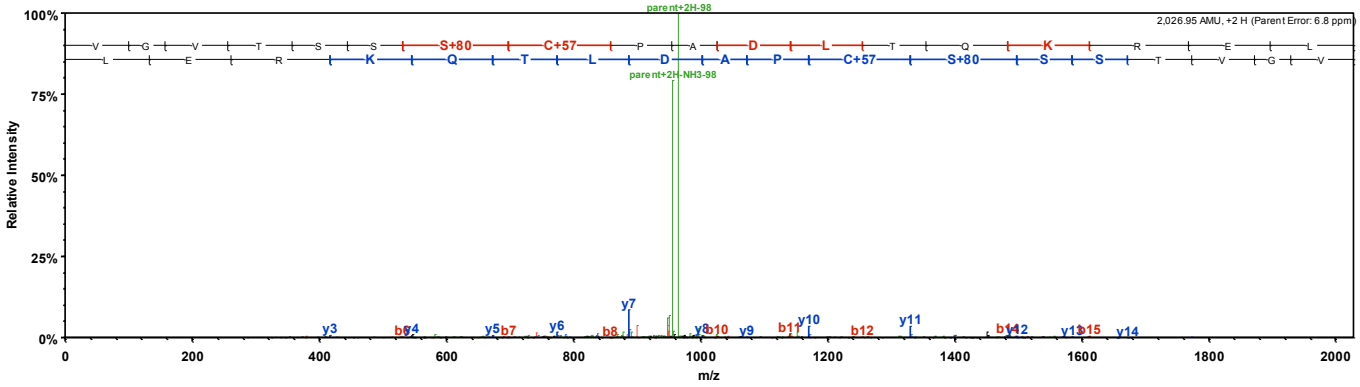
Predicted YWHA binding motifs

Site	Score	Sequence
S211	0.6162	LVGVTSS**S**CPADLTQ
S399	0.6164	HHLDFSH**S**LSFGGRE
S467	0.5341	KASSRRS**S**FSMEEGD

Figure S10. Prediction of YWHA binding motif in TFEB amino acid sequence. Putative YWHA binding motifs in human TFEB amino acid sequence were predicted using Scansite 2.0 (http://scansite.mit.edu.)[1], a computational algorithm that predicts protein–protein interactions and phosphorylation sites.

A

(L)VGVTSSscPADLTQKREL(T)



B

Label free quantitation:

Phosphoryl S211 peptide	Mass/charge ratio (m/z)	Charge (z)	Mascot ion Score	Chromatographic peak area		DMSO/ PP242
				DMSO	PP242	
VGVTSSp**S**CPADL	636.7659	2	49.18	1.79E+08	5.01E+07	3.57
VGVTSSp**S**CPADLTQKREL	1014.4840	2	49.62	7.71E+07	2.14E+07	3.61

Figure S11. LCMS results of phosphoryl S211 peptides. (A) CID spectrum of peptide VGVTSSp**S**CPADLTQKREL. **(B)** Label-free quantitation based on extracted ion chromatograms of both chymotryptic peptides showing decreased S211 phosphorylation in PP242 treated cells.

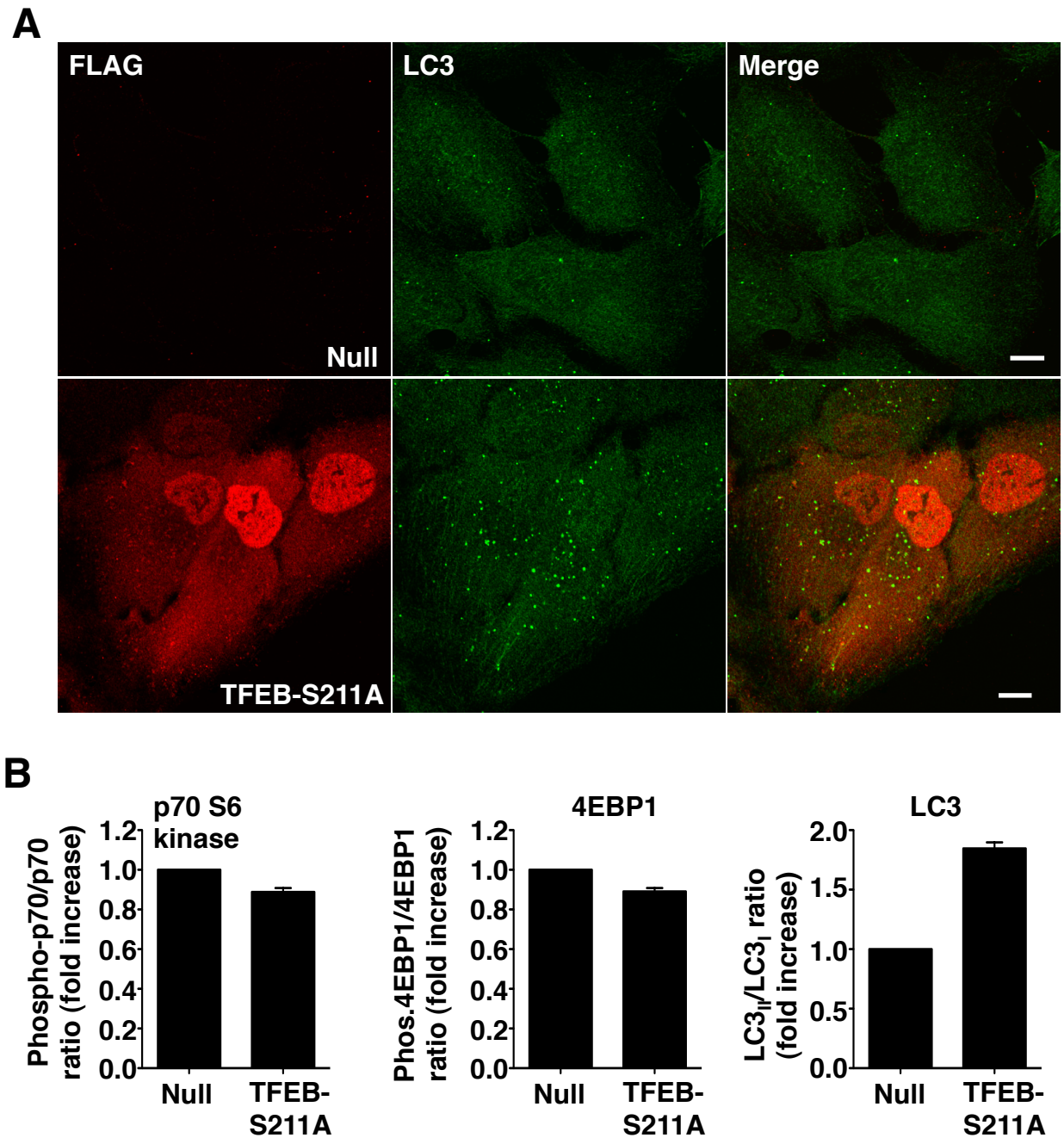


Figure S12. TFEB-S211A induces autophagosome formation in conditions where MTORC1 is active. ARPE-19 cells were infected with either Null adenovirus or adenovirus expressing TFEB-S211A-Flag. Fifteen hours after infection, cells were either fix in methanol:acetone for immunofluorescence (A) or lysed for immunoblotting (B). In **(A)** cells were double-stained with antibodies against Flag and LC3. Scale bars, 10 μm. In **(B)** cell lysates were subjected to immunoblotting analysis using antibodies against p70 S6 kinase, phospho-p70 S6 kinase, 4EBP1, phospho-4EBP1 and LC3. The corresponding protein bands were quantified using ImageJ software. Bars represent the ratio of phospho-p70 S6 kinase/p70 S6 kinase, phospho-4EBP1/4EBP1 and $LC3_{II}/LC3_{I}$ expressed as a fold increase of the ratio from cells infected with adenovirus Null. Values are means +/- SD of two independent experiments.

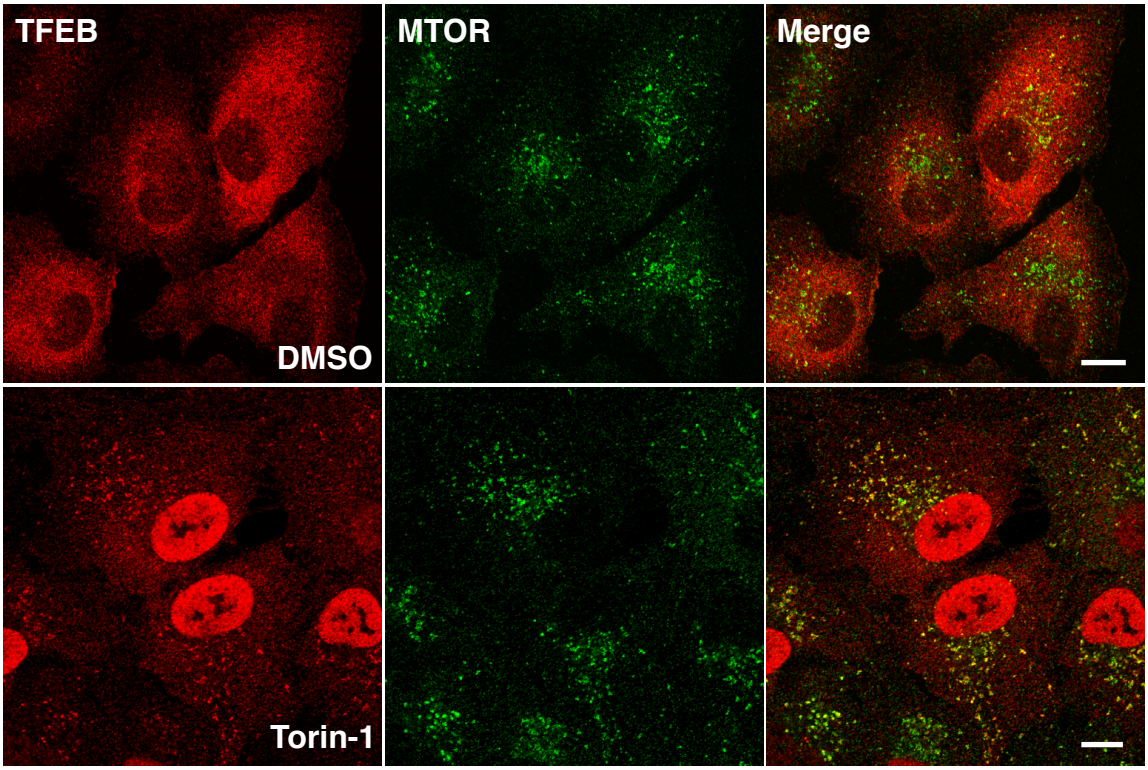


Figure S13. TFEB accumulates into the nucleus and is recruited to late endosomes/lysosomes upon MTORC1 inactivation by Torin-1. ARPE-19 cells were infected with adenovirus expressing TFEB-Flag. Fifteen hours after infection, cells were incubated with either DMSO (vehicle) or Torin-1 (250 nM) for 1 h. Cells were then washed, fixed, permeabilized with 0.5 % Triton X-100, and double-stained with antibodies against Flag and MTOR. Scale bars, 10 μm.